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Differential responses of macrophages to *Salmonella enterica* serovars Enteritidis and Typhimurium

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Abstract

Macrophages are major effectors against *Salmonella* infection, and also transport bacteria between host tissues and provide a protected site for intracellular bacterial replication. We hypothesized that differences in chicken macrophage responses to *Salmonella enterica* serovar Enteritidis (SE) and serovar Typhimurium (ST) played a role in preferential infection of eggs by SE compared with ST. To test this hypothesis, we determined bacterial phagocytosis and intracellular viability and macrophage nitric oxide (NO) production following in vitro infection with SE or ST in the presence or absence of interferon-γ (IFN-γ). The effects of bacterial components, lipopolysaccharide (LPS), outer membrane proteins (OMP) and flagella, on NO production were also assessed. Our results showed: (1) in the presence or absence of IFN-γ, the percentage macrophages phagocytizing SE and ST was similar; (2) the number of intracellular viable SE was significantly reduced compared with ST in the presence or absence of IFN-γ; (3) increased macrophage necrosis was seen in the presence of IFN-γ and ST; (4) *Salmonella* infection acted synergistically with IFN-γ in induction of nitric oxide production; and (5) in the absence of IFN-γ, macrophages produced significantly greater NO following treatment with SE outer membrane protein or flagella compared with ST OMP or flagella, while in the presence of IFN-γ significantly less NO was produced following

Abbreviations: GFP, green fluorescent protein; IFN-γ, interferon-γ; NO, nitric oxide; OMP, outer membrane protein; SE, *Salmonella enterica* serovar Enteritidis; ST, *Salmonella enterica* serovar Typhimurium

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treatment with SE-LPS compared with ST-LPS. These results suggest that differential responses of chicken macrophages to SE versus ST may result in increased macrophage death with ST, which could result in an increased inflammatory response as compared to SE.

Keywords: Interferon-γ; Nitric oxide; Food poisoning; Chicken; Egg

1. Introduction

Epidemiological studies have indicated that *Salmonella enterica* serovar Enteritidis (SE), in contrast to serovar Typhimurium (ST), is a major source of human food poisoning through consumption of contaminated eggs and egg products (Hopper and Mawer, 1988; Telzak et al., 1990; Altekruse et al., 1993; Henzler et al., 1994; CDC, 2000). Our recent studies showed that host responses to *Salmonella* is complex and involve many aspects of cell-mediated immunity (Babu et al., 2003, 2004). Underlying mechanisms involved in the transfer of *Salmonella* to eggs are not clearly understood (Lillehoj and Okamura, 2003). In our previous report, ovarian infection following systemic bacterial dissemination was related to SE-specific egg contamination (Okamura et al., 2001). While macrophages are major effecter cells eliciting innate immunity, they also transport *Salmonella* to host tissues (Vazquez-Torres et al., 1999), including the chicken reproductive tract (Okamura et al., 2001), and provide a protected site for intracellular bacterial replication (Richter-Dahlfors et al., 1997). Furthermore, different macrophage responses to distinct *Salmonella* serovars have been reported in human and murine settings (Schwan et al., 2000), but no reports have described a similar phenomenon in avian macrophages, except for the responses of chicken kidney cells against different serovars (Kaiser et al., 2000). Therefore, we hypothesized that differential macrophage responses in chickens against SE and ST may contribute to preferential SE infection of eggs.

Interferon-γ (IFN-γ) is produced by natural killer cells and T-lymphocytes and stimulates macrophages to secrete nitric oxide (NO), which forms peroxynitrite, a potent oxidant with anti-microbial properties (Lowenthal et al., 1995; Alam et al., 2002). Recombinant IFN-γ from both chickens and turkeys protected against SE infection in chickens (Farnell et al., 2001; Takehara et al., 2003) and the in vitro and in vivo effects of this cytokine on heterophil responses against *Salmonella* were documented (Kogut et al., 2002, 2003). However, the effects of IFN-γ on chicken macrophage function, particularly with regard to possible differential responses to SE and ST, remain to be evaluated. Therefore, the present investigation was conducted to compare various parameters of macrophage activity (phagocytosis, intracellular bacterial viability, and NO production) following in vitro infection with SE and ST and to determine the effects of IFN-γ on these activities.

2. Materials and methods

2.1. Peripheral blood mononuclear cell (PBMC)-derived macrophages

PBMC-derived macrophages were isolated and cultured as described by Kaspers et al. (1994) with the following modifications. Heparinized blood was collected by cardiac puncture from 3- to 7-month-old White Leghorn chickens (SC inbred strain, Hyline, Dallas Center, IA). To obtain optimum numbers of macrophages, 3–4 chickens were bled and their pooled blood was used to isolate macrophages. Erythrocytes and heterophils were removed by sequential density gradient centrifugations using 1-Step™ Polymorphs (Accurate Chemical, Westbury, NY) and Histopaque®-1077 (Sigma, St. Louis, MO) according to the manufacturers' instructions. These steps provide efficient removal of heterophils and erythrocytes, respectively. Cell viability was >95% by Trypan blue exclusion. The cells were resuspended at 2.0×10^7 cells/ml in RPMI-1640 with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin, seeded into 75 cm^2 culture flasks or 100 mm Petri dishes, and incubated at 41 °C in 5% CO_2. Fresh culture medium was added at 24 h, the remaining cells washed with

Hanks' balanced salt solution (Sigma) at 48 h, and adherent PBMC-derived macrophages collected by treatment with 0.5 mM EDTA for 10–30 min at 41 °C. Purity of macrophages was >80% as determined by flow cytometric analysis using the monoclonal antibody against K1, the chicken macrophage surface marker (Chung and Lillehoj, 1991).

2.2. In vitro Salmonella infection

Infection of macrophages with *Salmonella* was performed as described by Raybourne et al. (2001) with modifications. PBMC-derived macrophages were washed with Hanks' balanced salt solution, resuspended to 1.0×10^6 cells/ml in antibiotic-free RPMI-1640 with 10% FCS, and exposed to green fluorescence protein (GFP)-expressing SE strain 338 or GFP-ST strain SR11 (Miller and Lindow, 1997) at a multiplicity of infection of 20–100 in the presence or absence of IFN-γ. Chicken recombinant IFN-γ was prepared from culture supernatants of COS7 cells stably transfected with the corresponding cDNA (Lillehoj and Choi, 1998) and used at a final dilution of 1:50. Since there is no standardized commercial recombinant chicken IFN-γ available, the dilution of recombinant chicken IFN-γ that we used in this study was chosen on the basis of its maximum effect on NO production.

The cells were incubated with continuous rotation for 2 h at 41 °C in 5% CO_2, washed two times in RPMI-10 supplemented with 100 μg/ml gentamycin, and incubated for 0, 6, 12, or 24 h. For negative controls, cells were non-infected or treated with supernatant of vector-transfected COS7 cells at the same dilution as the supernatant of IFN-γ-transfected cells.

2.3. Salmonella phagocytosis and intracellular bacterial viability

Post-infection, the GFP-positive cells were analyzed by flow cytometry (Coulter Beckman, Miami, FL) to assess the percentage of cells containing intracellular bacteria. To determine the viability of intracellular bacteria, infected macrophages were sorted in triplicate in 96-well tissue culture plates containing 100 μl/well of 0.01% Triton X-100 (100 cells/well). The cells were incubated for 20 min at room temperature, lysates plated on trypticase soy agar (Difco, Detroit, MI), and CFU enumerated.

2.4. Macrophage apoptosis

Analysis of phosphatidylserine on the outer membrane of macrophages undergoing apoptosis was performed with the annexin V-PE detection kit according to the manufacturer's instructions (Becton Dickinson Biosciences, San Diego, CA). Briefly, cells were uninfected or infected with SE or ST as described above and 1.0×10^6 cells in 50 μl incubated with 5.0 μl of annexin V-phycoerythrin and 5.0 μl of 7-amino-actinomycin D (7-AAD) reagents for 15 min at room temperature. Cells were analyzed by flow cytometry as above.

2.5. NO production

Culture supernatants of SE- or ST-infected macrophages were collected at 2, 6, 12, and 24 h post-infection, clarified by membrane filtration (0.45 μm, Schleicher & Schuell, Keene, NH), and analyzed for NO using Griess reagent (Sigma). The absorbance was measured at 540 nm and the nitrite concentration determined using a standard curve generated with sodium nitrite.

2.6. Treatment of macrophages with bacterial products

Non-infected cells were treated for 24 h with SE or ST outer membrane protein (OMP), LPS, or flagella at 20 μg/ml in RPMI-1640 with 10% FCS in the presence or absence of IFN-γ. LPS preparations of SE and ST were obtained from Sigma (cat. nos. L7770 and L6143). OMP and flagella were prepared as described (Okamura et al., 2003). Briefly, overnight cultures of SE strain FDA338 and ST strain SR11 in trypticase soy broth supplemented with yeast extract were collected by centrifugation at $4500 \times g$ for 30 min at 4 °C, washed with PBS, and adjusted to 10^9–10^{10} CFU/ml. To prepare OMP, bacteria were sonicated for 3 min on ice, centrifuged at $1500 \times g$ for 30 min, the supernatant centrifuged at $20{,}000 \times g$ for 30 min, and the pellet collected. To prepare flagella, bacteria were homogenized at

30,000 rpm for 1 min on ice (OMNI International, Warrenton, VA), centrifuged at 2000 × *g* for 30 min, and the supernatant collected. Protein concentrations were determined by the method of Lowry et al. (1951).

2.7. Statistical analysis

All experiments were performed in triplicate in three independent sets, differences between means determined by one-way ANOVA, and considered significant at $P < 0.05$.

3. Results

3.1. Salmonella phagocytosis and killing by macrophages

To compare the ability of chicken macrophages to phagocytize SE and ST, and the influence of IFN-γ on this process, isolated PBMC-derived adherent cells were infected for 2 h with GFP-SE or GFP-ST in the presence or absence of recombinant IFN-γ, washed with gentamycin to kill extracellular bacteria and analyzed by flow cytometry for the presence of

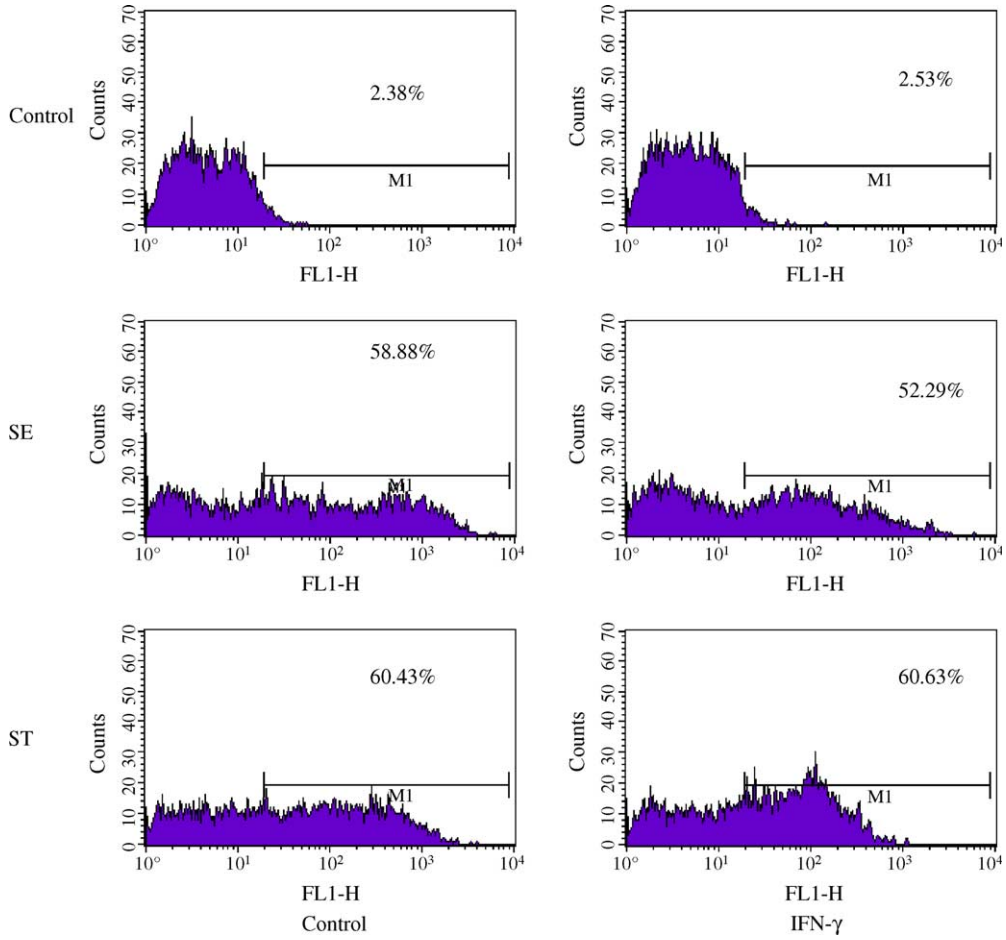

Fig. 1. *Salmonella* phagocytosis by macrophages. Macrophages were infected for 2 h with GFP-SE or GFP-ST in the presence or absence of IFN-γ, washed, and analyzed for intracellular bacteria by flow cytometry. Non-infected control cells are also shown. The *x*-axis of each histogram is relative GFP fluorescence (log scale), the *y*-axis is number of cells. Marker regions (M1) indicated the area designated as positive for GFP, with the percentage of cells in that region shown. These regions also approximate the fluorescence gates used for sorting and bacterial enumeration. Results are representative of three experiments.

intracellular bacteria (Fig. 1). At 0 h, the percentage of infected primary macrophage following incubation with bacteria was similar for ST and SE, with and without IFN-γ. The mean fluorescence intensity of infected cells was lower for ST, due to the fact that GFP expression in this ST strain is less than in SE. Macrophages containing intracellular ST and SE were easily distinguishable from non-infected cells by flow cytometry (Fig. 1). Cell sorting regions were established that included the entire infected cell population for both ST and SE. Because the entire infected cell population was included in the sort region, all such cells were considered positive and were randomly sorted without regard to relative fluorescence. Therefore, the difference in fluorescence between GFP SE and ST would not influence the results. We determined the number of viable bacteria within macrophages by sorting a constant number of GFP-positive cells, subjecting them to detergent lysis, and counting the number of released CFU on trypticase soy agar (Fig. 2). In the absence of IFN-γ, both serovars increased over a 12 h incubation, albeit with different levels of efficiency. The number of ST increased to a greater degree than seen with SE ($P < 0.001$). Addition of IFN-γ resulted in significantly reduced numbers of both SE and ST at 12 h ($P < 0.01$ and $P < 0.0005$, respectively). Relative to SE, there was greater recovery of ST with or without IFN-γ, indicating more intracellular replication by ST and/or more resistance to killing ($P < 0.05$).

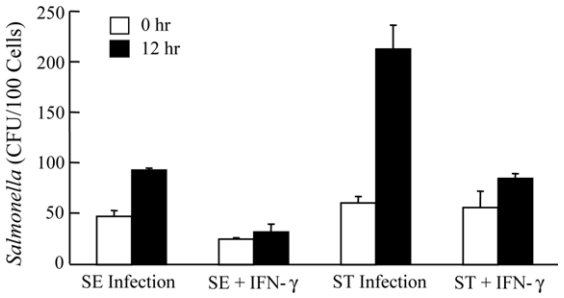

Fig. 2. *Salmonella* intracellular viability. Macrophages were infected for 2 h with GFP-SE or GFP-ST in the presence or absence of IFN-γ, washed, isolated by cell sorting at 0 or 12 h post-infection, lysed in detergent solution, and viable bacteria enumerated on trypticase soy agar. The data are expressed as the number of CFU per 100 macrophages and represent the mean ± S.D. of triplicate determinations.

3.2. *Salmonella*-induced macrophage apoptosis

Salmonella infection induces apoptosis of mouse primary macrophages and macrophage-like cell lines (Monack and Falkow, 2000; Monack et al., 2001a,b). To determine if apoptosis occurs in bacterially-infected primary avian phagocytes, and the effect of IFN-γ on this process, macrophages infected with GFP-SE or GFP-ST were exposed to PE-conjugated annexin V to detect membrane exposure of phosphatidylserine (PS) which is associated with early stages of apoptosis and 7-amino actinomycin D which

Table 1
Percentage of infected cells falling into three populations based on dual fluorescent staining with phycoerythrin labeled annexin V and 7-amino actinomycin D

	Time (h)							
	0		6		12		24	
	−[a]	+[a]	−	+	−	+	−	+
SE-infected								
Annexin+/7-AAD− "APOPTOTIC"	44.8	50.8	61.6	56.1	72.1	58.0	64.0	51.5
Annexin+/7-AAD+ "NECROTIC"	24.1	23.0	13.2	16.5	9.6	21.1	16.6	18.0
Annexin−/7-AAD− "VIABLE"	27.3	23.9	24.8	25.0	18.3	20.1	19.1	28.8
ST-infected								
Annexin+/7-AAD− "APOPTOTIC"	45.5	49.2	66.3	50.2	72.7	53.5	61.4	44.7
Annexin+/7-AAD+ "NECROTIC"	27.1	23.7	12.3	23.9	12.3	25.5	19.0	26.9
Annexin−/7-AAD− "VIABLE"	24.9	25.8	21.3	23.7	14.9	19.6	19.4	23.9

This table shows a representative result from two independent experiments. Values based on analysis of 5000 cells. Cells with surface exposed phosphatidylserine (annexin+) and non-permeable cell membrane (7-AAD−) characteristic of early apoptosis. Cells with exposed phosphatidylserine (annexin+) and permeable cell membrane (7-AAD−) characteristic of late apoptosis or necrosis.

[a] IFN-γ.

detects compromised membrane integrity. Cells were analyzed by flow cytometry for GFP, PE, and 7-AAD staining. In order to confine the apoptosis analysis to cell with intracellular bacteria, a gate was established around the GFP positive population, and these cells were analyzed for PE-annexin V and 7-AAD staining (Table 1). Three populations of cells were apparently based on PE-annexin V and 7-AAD staining: early apoptotic cells stained with PE-annexin V only (PS+); membrane compromised (necrotic or late apoptotic) stained with PE-annexin V and 7-AAD; and cells negative for both PE-annexin V and 7-AAD (Table 1). Because all 7-AAD positive cells were also positive for PE-annexin V, an increase in the percent necrotic population was accompanied by a decrease in the early apoptotic population. Comparison across time points was also difficult because percentages can be affected by necrotic cells that disintegrate and thus become undetectable at subsequent points, for example, between 0 and 6 h. In the absence of IFN-γ, ST and SE infected phagocytes were similar in their percentages of early apoptotic and membrane compromised cells at 0 and 6 h and tended to increase more with ST at 12 and 24 h. This effect was magnified by exposure to IFN-γ and was most pronounced with ST at 6 and 24 h.

3.3. Macrophage NO production in response to SE or ST infection

Generation of NO is an essential feature of inflammation in response to *Salmonella* infection (Alam et al., 2002). Therefore, we examined NO levels in culture supernatants of macrophages at various times post-infection with SE or ST in the presence or absence of IFN-γ. Exposure of non-infected cells to IFN-γ induced production of NO over time compared with controls (Fig. 3). With infected cells in the absence of IFN-γ, NO levels in SE- and ST-infected macrophages were equivalent, with the exception of the 2 h time point. SE- and ST-infected macrophages treated with IFN-γ showed equivalently increased NO production at 24 h compared to controls without IFN-γ ($P < 0.0005$). At 2 h post-infection, SE- or ST-infected cells with IFN-γ showed lower levels of NO production than those without the cytokine ($P < 0.001$ and $P < 0.05$, respectively).

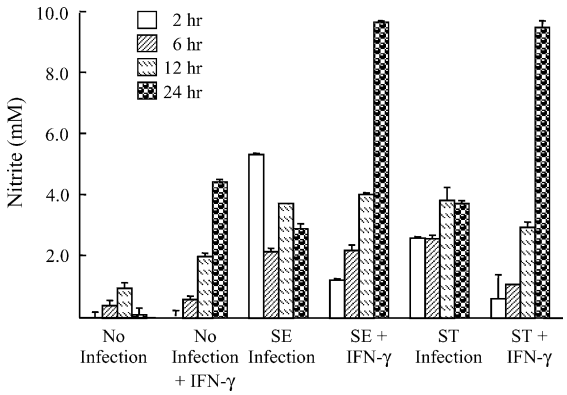

Fig. 3. Macrophage NO production in response to SE or ST infection. Macrophages were infected for 2 h with SE or ST in the presence or absence of IFN-γ, washed, incubated for 2, 6, 12, or 24 h, and NO levels in culture supernatants determined. Values represent the mean ± S.D. concentrations of nitrite from triplicate determinations.

3.4. Macrophage NO production in response to bacterial products

We previously reported that *Salmonella* OMP, LPS, and flagella were capable of eliciting various components of protective immunity to bacterial infection when administered in vivo to chickens (Okamura et al., 2003, 2004). Therefore, we next

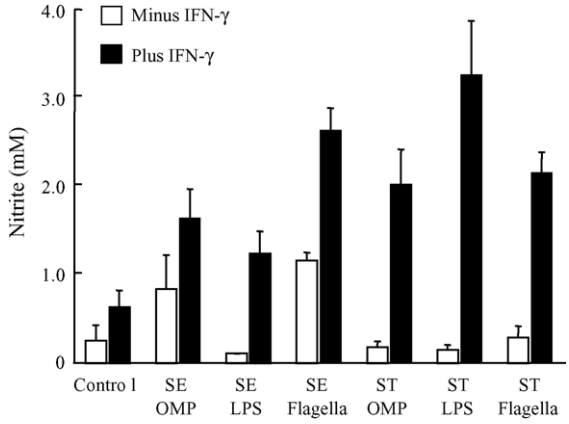

Fig. 4. Macrophage NO production in response to bacterial products. Macrophages were treated with 20 μg/ml SE or ST OMP, LPS, or flagella for 24 h in the presence or absence of IFN-γ and NO levels in culture supernatants determined. Values represent the mean ± S.D. concentrations of nitrite from triplicate determinations.

examined the ability of these bacterial components isolated from SE or ST to stimulate NO production by macrophages in vitro in the presence or absence of IFN-γ. As shown in Fig. 4, in the absence of IFN-γ, macrophages produced significantly more NO following treatment with SE OMP or SE flagella compared with ST OMP or ST flagella, respectively ($P < 0.05$). There were no differences in NO levels following treatment with LPS from SE versus ST. In contrast, in the presence of IFN-γ, macrophages produced significantly less NO following treatment with SE LPS compared with ST LPS ($P < 0.05$) while the effects of OMP and flagella from the two *Salmonella* serovars were equal.

4. Discussion

This study demonstrated that macrophages responded differently to infection with SE or ST with respect to intracellular bacterial viability, induction of apoptosis/necrosis, and NO production. In addition, these activities were influenced to different degrees by IFN-γ depending on the infecting bacterial serovar. With respect to phagocytosis, uptake of SE and ST by primary phagocytes was equivalent with or without IFN-γ as determined by flow cytometry. This parallel-plate counts from sorted, infected cells at 0 h, where no significant differences occurred. In the absence of IFN-γ, ST exhibited more robust intracellular replication by 12 h of infection. IFN-γ treatment enhanced killing of both ST and SE by phagocytes, with ST showing greater survival. With respect to NO production, treatment with SE in the absence of IFN-γ induced greater NO levels at 2 h post-infection compared with ST, however, this trend was not observed at other time points, making it difficult to determine its biological significance. Without IFN-γ, SE OMP or flagella increased NO production compared with the corresponding ST bacterial products. In the presence of IFN-γ, NO levels induced by SE LPS were reduced compared with ST LPS.

It is tempting to speculate that different macrophage responses to SE and ST infections may be related to the persistent systemic infection and egg contamination associated with the former serovar (Okamura et al., 2001). ST to a greater extent than SE, in combination with IFN-γ treatment induced increased cellular membrane permeability, a marker for late apoptotic or necrotic cells. This suggests a difference in the degree of macrophage necrosis induced by ST versus SE. Cell death pathways resulting in apoptosis or necrosis can have differing outcomes relative to the inflammatory response. Apoptosis tends to be less inflammatory than necrosis, which is strongly proinflammatory and can be induced by *Salmonella* in a Caspase 1 dependant process (Brennan and Cookson, 2000). An in vivo consequence of this might be to induce a more vigorous and acute inflammatory response to ST, resulting in rapid bacterial clearance, while SE infection may induce less inflammation resulting in a more commensal, long lasting infection with few overt effects on the health of chickens. In contrast, ST infection can be cleared more rapidly by induction of inflammatory responses, which may further activate Toll-like receptors (Bihl et al., 2003).

The increase in NO production following phagocytosis of bacteria is synergized by IFN-γ in both ST and SE infection. In addition, bacterial OMP-, flagella- and LPS-induced NO production was also enhanced by IFN-γ. In the presence of IFN-γ, ST-LPS induced more NO than SE-LPS. LPS has been shown to elicit NO production in mice, particularly in combination with IFN-γ (Ding et al., 1998). In studies with chicken macrophage cell lines, NO production was linked to apoptosis and necrosis induced by CpG oligodeoxynucleotides (Xie et al., 2003). Increased NO levels induced by ST and ST-LPS in conjunction with IFN-γ may be related to increased apoptosis and necrosis observed in our study.

In conclusion, our results indicate that chicken macrophages displayed differences in their responses to SE and ST, which might contribute to the differential pathogenesis of these *Salmonella* serovars. In general, macrophage responses were enhanced and intracellular viable bacteria were reduced by IFN-γ. It is possible that reduced IFN-γ production by chicken heterophils in response to SE infection (Kogut et al., 2003; Okamura et al., 2004) leads to more systemic infection and egg contamination. Further elucidation of the molecular and cellular events surrounding *Salmonellae*, macrophages, cytokines, and chemokines will provide a better understanding of the mechanisms underlying SE egg contamination.

Acknowledgments

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